

# SOME NOTES ON FLUORESCENCE INTENSITY

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**ABSTRACT** The fluorescent emission spectra for  $5 \times 10^{-6}$  M *p*-aminohippuric and *p*-aminobenzoic acids in mixtures of methyl alcohol and 1,2-propanediol have been determined. The results indicate that at almost invariant dielectric constant the quantum yield of fluorescence is a function of the viscosity of the solvent. The suggestion is made that a collisional quenching mechanism, which involves the rotational diffusion of solvent molecules, is significant in solutions of low viscosity and less so at high viscosities. A prediction, on the basis of the proposed mechanism, of augmentation of the emission spectra of *p*-aminohippuric acid, after binding to homologous antihapten antibody, is confirmed. A small red shift is also noted at higher viscosities in protein-free solutions or after binding to homologous antibody. It is suggested that, contrary to some interpretations in the literature, a red shift and/or an augmentation of quantum yield of fluorescence may, in specific instances, not be significant of a transfer of the fluorochrome to an environment of lower dielectric constant.

## INTRODUCTION

In some recent reports, it has been observed that the complexing of a small, slightly fluorescent molecule to a macromolecule results in an increased quantum yield of emitted fluorescence ( $Q$ ) (1-5).<sup>1</sup> The suggestion has been made that augmented  $Q$  for some fluorochromes ( $F$ ) in solution in the absence of macromolecules may be associated with an increase in the macroscopic viscosity ( $\eta$ ) of the solvent; this in turn has been correlated with an increase in  $\eta$  in the immediate environment of  $F$  (6). The mechanism by which  $\eta$  modifies  $Q$  may also operate at protein binding sites if an effective viscosity for the binding site is assumed (1). As a class, the type of fluorochrome which seemed to be characterized by a positive relation between  $Q$  and  $\eta$  has at least one degree of intramolecular rotational or torsional freedom between relatively bulky moieties which are themselves rigid.

We now report on the effect of the large variations of  $\eta$  at almost invariant dielectric constant ( $D$ ), on two molecules which lack the structural feature of the

<sup>1</sup> Abbreviations used: BGG, bovine gamma globulin;  $D$ , dielectric constant;  $\eta$ , viscosity;  $F$ , fluorochrome in its electronic ground state;  $F'$ , fluorochrome in its first excited electronic state; PAB, *p*-aminobenzoate; PABH, *p*-aminobenzoic acid; PAH, *p*-aminohippurate; PAHH, *p*-aminohippuric acid; RGG, rabbit gamma globulin.

class defined above. Our measurement of the emission spectra of  $5 \times 10^{-6}$  M *p*-aminohippuric acid (PAHH) or *p*-aminobenzoic acid (PABH) in a series of solvent mixtures established the fact that an increase in viscosity at almost constant *D* is accompanied by both a red shift in the emission spectra and an increased *Q* (Fig. 2). The binding of *p*-aminohippurate (PAH) to its purified homologous anti-hapten antibody also induces qualitatively similar changes in the emission spectra of PAH (Fig. 4). The results discussed in this paper suggest that:

1. Collisions between the solvent molecules and the excited fluorochrome (*F'*) may result in a radiationless path to the ground state for a significant number of molecules.

2. Some fraction of the increase in *Q* noted when *F* is allowed to complex with macromolecules, may be due to steric interference which prevents or inhibits collisional quenching of *F'* by solvent molecules.

## MATERIALS AND METHODS

PABH and PAHH were obtained from Fisher Scientific Company, Pittsburgh, Pa. and twice recrystallized from ethanol. They were dissolved in borate buffer pH 8. I = 0.16 (borate) or in Fisher "Spectroanalyzed" grade methanol to make 0.01 M stock solutions.

All solvents were obtained commercially and twice redistilled; 1,2-propanediol was distilled at reduced pressure. Immediately prior to use the solvents were brought to their respective boiling points, maintained there for 10 min, allowed to cool, and subsequently flushed with nitrogen.

Antisera were produced by a series of triweekly intravenous injections of PAHH which had been diazotized and coupled to bovine- $\gamma$ -globulin (BGG). High titer antisera were pooled and precipitated by bringing the sera to 15% sodium sulfate. The precipitates were redissolved in borate and concentrations were adjusted to 10 mg protein/ml ( $1 \times$  fractionated globulin).

Antibodies were purified with the aid of a solid adsorbent prepared by precipitation, at a slight antigen excess, of the BGG azohapten injecting antigen with anti-BGG. After 5 days at 2°–5°C, the adsorbent was separated by centrifugation and washed with borate until the OD at 280 m $\mu$  of the borate wash was lower than 0.025. 25 ml of  $1 \times$  fractionated globulin was adsorbed overnight at 2°–5°C with a suspension of the adsorbent prepared from 30 mg of BGG-azohapten. Subsequently the adsorbent was washed three times with cold borate and the antibody eluted with 0.1 M hapten in borate. A major portion of the hapten was separated from the antibody by dialysis and the remainder removed by passage through a strong anionic exchange resin (Amberlite GC 400) (Mallinckrodt, N. Y.) in a 5 ml syringe. The eluted antibody was more than 85% precipitable with the homologous hapten coupled to rabbit- $\gamma$ -globulin (RGG).

The basic fluorimeter used had been previously described (7). Studies involving mixed solvents or proteins were undertaken with a modification in which a unit voltage gain, solid state, impedance matching amplifier was interposed between the photomultiplier and the recorder. This modification resulted in a large power gain and made it possible to operate at narrower slit widths and lower photomultiplier voltages. Excitation and emission monochromators were operated at a band pass of 0.8 m $\mu$ . A second 1P28 photomultiplier intercepted the exciting radiation after it had traversed the sample cuvette. The output of this photomultiplier was read directly on a vacuum tube voltmeter. The readings were used to correct the magnitude of the spectral curves in accordance with long term

fluctuations in the exciting light intensity. A Baird Fluorospec Spectrophotofluorimeter (Baird Instruments, Cambridge, Mass.) operated at a  $32\text{ m}\mu$  band-pass was used for determinations of spectra in pure alcohols. The OD of all solutions, at the exciting wavelength, was never allowed to exceed 0.08; consequently for protein containing solutions, cells with a 3 mm path length had to be used.

A Cary model 11 or a Cary model 14 (Applied Physics Corp., Monrovia, Calif.) was used for spectrophotometric determinations. The Cary 14 was equipped with a 0 to 0.1 OD slide wire.

Relative quantum yields were determined by planimetry of recorded emission spectra after adjustment to constant absorption at the exciting wavelength.

Viscosities were measured in Ostwald viscometers (Greiner Scientific Corp., N. Y.) thermostated at  $23^\circ\text{C}$ .

Equilibrium dialysis was performed in duplicate with 1 ml of purified antibody or normal RGG inside Visking tubing (Greiner) against a large volume of  $5 \times 10^{-6}\text{ M}$  tritium-labeled PAH in borate. The labeled compound was obtained from New England Nuclear Corp., Boston, Mass., and used without further purification.

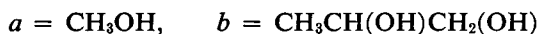
## RESULTS AND DISCUSSION

PAB and PAH are detectably fluorescent in aqueous solution and increase their fluorescence severalfold when dissolved in the more viscous, less polar aliphatic alcohols. As the solvent is changed in the sequence, methanol, etha nol, 1-propanol 1-butanol,  $Q$  increases but both  $D$  and  $\eta$  change at the same time (Table I). Consequently, in order to evaluate the effect of a change in only one parameter it was decided to employ a mixed solvent system in which  $\eta$  could be varied independently of  $D$ .

The system chosen consisted of mixtures of methyl alcohol ( $D = 33$ ,  $\eta_{23} = 0.59$ ) and 1,2-propanediol ( $D = 32.5$ ,  $\eta_{23} = 33$ ). This solvent system makes available an almost sixtyfold variation in  $\eta$ . The two pure solvents have almost identical dielectric constants, but the possibility of anomalous variations in  $D$  for some ratios of solvents does exist. No means were available for a direct evaluation of this possibility. However, some evidence for the formation of ideal solutions from mixtures of these components was obtained through the experimental determination of  $\eta$  for an aliquot of each of the solvent mixtures. Values for the pure solvents were substituted in equation 1, which describes the viscosities of ideal binary solutions in terms of their composition and the viscosity of each of the pure components:

$$\log 1/\eta = X_a \log 1/\eta_a + X_b \log 1/\eta_b \quad (1)$$

$X$  = mole fraction



In accord with this equation a plot of  $\log 1/\eta$  vs. the mole fraction of methyl alcohol would be a straight line. As can be seen from Fig. 1, by this criterion, solutions

formed from all ratios of methyl alcohol to 1,2-propanediol behave in an ideal fashion.

The emission spectra of  $5 \times 10^{-6}$  M PAH, excited at  $290 \text{ m}\mu$  in methyl alcohol-1,2-propanediol solvent systems are reproduced in Fig. 2. The mole fraction of the diol has been varied from zero to one in steps of 0.2. Evident from an inspection of the figure is the fact that as the mole fraction of the diol increases, so does  $Q$ .  $D$  is virtually constant, and if there is no unique interaction between either of

TABLE I  
THE QUANTUM YIELD OF FLUORESCENCE OF  $5 \times 10^{-6}$  M P-AMINOBENZOIC ACID (PABH) OR P-AMINOHIPURIC ACID (PAHH)  
IN VARIOUS ALCOHOLS

Fluorescence was excited at  $290 \text{ m}\mu$

Solvent	Dielectric constant at $25^\circ$	Viscosity at $20^\circ$ (centipoise)	Relative quantum yield at constant absorption	
			PABH	PAHH
Methanol	32.6	0.597	1	1
Ethanol	24.3	1.200	1.17	1.44
Propanol	20.1	2.256	1.51	1.52
Butanol	17.1	2.95	1.93	1.69

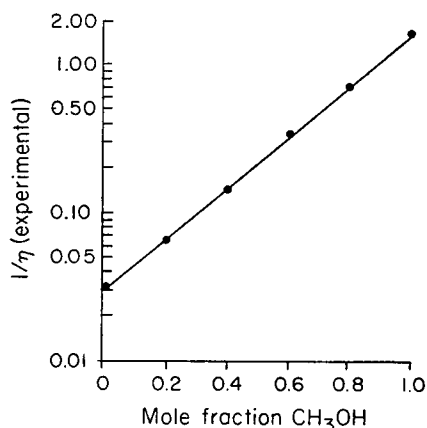


FIGURE 1 The log of the reciprocal of the experimentally determined resultant viscosity as a function of the mole fraction methanol in methyl alcohol, 1,2-propanediol mixtures. The straight line relationship is characteristic of ideal solutions.

the solvent components and  $F'$ , it appears reasonable to implicate  $\eta$  in the increase in  $Q$ .

Some suggestive evidence for the absence of any unique interaction between either of the pure solvents and  $F'$  can be obtained by a comparison of the ratio of the quantum yield to the height of the emission peak in each of the solvent systems used. The ratio is constant (Table II) but emission takes place from the lowest vibrational level of the first excited electronic state to higher vibrational levels in the ground state without a change in the conformation peculiar to  $F'$

(Frank-Condon principle). Therefore if any interaction with  $F'$ , unique to only one of the solvents, was present, the spacing of vibrational levels would be expected to be altered and, consequently, the shape of the emission curve would be altered. In that case the ratio defined above would not have been constant unless compensatory changes in shape and magnitude occurred.

It has become almost conventional to assume that a positive relationship be-

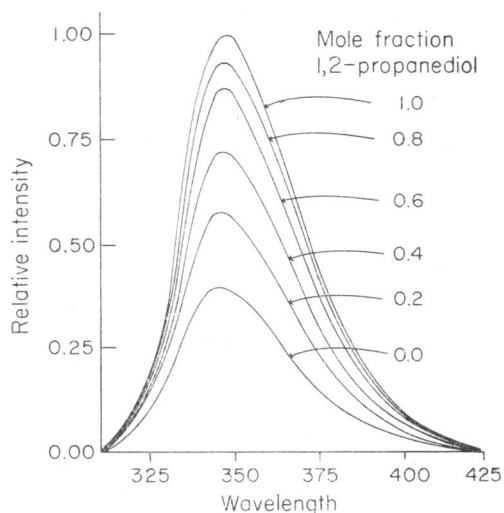


FIGURE 2 Emission spectra of  $5 \times 10^{-6}$  M *p*-aminohippuric acid in a solvent system composed of methyl alcohol and 1,2-propanediol. Evidence is presented that both the dielectric constant of the solvent mixtures and the shape of the curves are almost invariant.

TABLE II  
THE QUANTUM YIELD AND THE MAGNITUDE OF THE EMISSION MAXIMA  
OF THE CURVES REPRODUCED IN FIG. 2

Each set of values is relative to the value obtained in pure propanediol

Mole fraction 1,2-propanediol	Quantum yield (relative)	Maximum emission intensity (relative)
1	1	1
0.8	0.96	0.94
0.6	0.88	0.87
0.4	0.71	0.72
0.2	0.57	0.58
0.0	0.39	0.38

tween  $\eta$  and  $Q$  is the result of an inhibition of intramolecular rotation by high viscosity. It is unlikely that viscosity changes would influence intramolecular rotations involving a small amino group. Therefore if the above assumption is correct, in the present instance, the fluorescence of the similar molecule PABH should be less sensitive to changes in viscosity than PAHH. This conclusion is based on the fact that PABH lacks the bulkier glycyI residue of PAHH. To test

this conclusion emission spectra for PABH in the same solvent system were determined. The ratio of peak heights in 1,2-propanediol to that in methyl alcohol is 4.5 for PABH and only 2.6 for PAHH. Therefore it seems that PABH is actually more sensitive to changes in viscosity than PAHH, and consequently it is unlikely that inhibitions of intramolecular rotations are a significant factor in the observed effects.

A mechanism consistent with the above data can be postulated on the basis of an assumed intersection between the multidimensional potential surfaces which would describe the ground and first excited electronic levels of  $F$ . These surfaces which represent potential energy as a function of the spatial distribution of all the atoms forming  $F$  or  $F'$ , must be assumed to cross at values for vibrational and rotational states of  $F'$  which are not heavily populated at room temperature. The

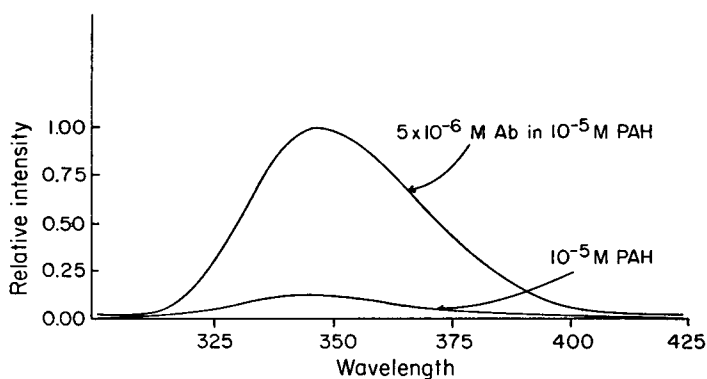


FIGURE 3 The emission spectra of *p*-aminohippurate in pH 8 borate buffer alone or in the presence of  $5 \times 10^{-6}$  M specifically purified homologous antihippurate antibody. The spectra were excited at  $300 \text{ m}\mu$  and the solutions were contained in square cells with a 3 mm path length. Equilibrium dialysis had established the fact that one-half of the hapten is bound under the conditions of the experiment.

population of these states may be increased by transfer of energy during a collision. Subsequently, by a process of tunneling, without a change of nuclear coordinates, the fluorochrome may go from an excited electronic state with a moderate amount of vibrational excitation to the ground electronic level with higher vibrational excitation. This would be a radiationless transition and the vibrational excitation of the electronic ground state may be quickly lost by thermal relaxation.

It is probably true that a specific orientation between  $F'$  and a quenching solvent molecule must be realized before the requisite energy transfer can take place. This may require rotational as well as translational diffusion over a few molecular diameters. However, the rotational diffusion constants are inversely proportional to viscosity. If, for quenching to occur, a specific rotation is required during the mean lifetime of fluorescence, the higher the viscosity the lower the probability

of quenching. Therefore, qualitatively at least, the observed relation between  $Q$  and  $\eta$  would result.

If the mechanism proposed above is correct, it might be expected that the complexing of PAH with a macromolecule would also augment  $Q$  since the presence of the macromolecule would interfere with the access of solvent molecules to  $F'$ . Emission spectra for PAH in borate and for PAH complexed with purified antibody were determined at 300 m $\mu$  excitation. This excitation wavelength was selected in order to reduce possible energy transfer contribution to the magnitude of the emission spectra. Equilibrium dialysis of a large value of  $5 \times 10^{-6}$  M tritium-labeled PAH against  $5 \times 10^{-6}$  M purified homologous antibody established the fact that approximately half the hapten was bound in the experiment described above. The results are plotted in Fig. 3 in which an eight- to ninefold increase in emission maximum is realized. This observation, to some degree, confirms the prediction which results from the assumption of a collisional quenching mechanism for deactivation.

Careful examination of the curves reproduced in Fig. 2 enables one to discern a small but real red shift ( $1.6 \pm 0.8$  m $\mu$ ) accompanying an increase in  $\eta$ . A similarly small, but real red shift also results when PAH is allowed to complex with purified anti-PAH. The significance of these two observations is not at present clear. The effect noted, whatever the mechanism, does not seem to affect the fact that the ratio of the peak heights to the integrated areas of the emission curves is constant within experimental error.

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